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(71) Applicant *(for all designated States except US)*: CHR. HANSEN A/S [DK/DK]; Bøge Allé 10–12, DK–2970 Hørsholm (DK).

(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: NILSSON, Dan [DK/DK]; Gefionsbakken 4A, DK–3060 Espergærde (DK). JANZEN, Thomas [DK/DK]; Ingemannsvej 30, 3. tv., DK–1964 Frederiksberg (DK).

(74) Agent: PLOUGMANN, VINGTOFT & PARTNERS; Sankt Annæ Plads 11, P.O. Box 3007, DK–1021 Copenhagen K (DK).

(54) Title: METHOD OF PREVENTING BACTERIOPHAGE INFECTION OF BACTERIAL CULTURES

(57) Abstract

    Method of preventing bacteriophage infection of bacterial cultures comprising modified strains, wherein the cultures are completely resistant to bacteriophage attack and have retained their capability of being metabolically active. The method is useful in the manufacturing of food products, feed products or useful metabolite products.

METHOD OF PREVENTING BACTERIOPHAGE INFECTION OF BACTERIAL CULTURES

FIELD OF INVENTION

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The present invention relates to the field of bacterial cultures which are used industrially in the manufacturing of e.g. food products or useful metabolite products. In particular there is provided modified bacterial strains, which, when cultivated in a selected substrate material, are not susceptible to attack by bacteriophages, and have

10 retained their capability of being metabolically active.

TECHNICAL BACKGROUND AND PRIOR ART

15 Production failures of bacterial cultures caused by bacteriophage infection are considered to be one of the major problems in industrial use of bacterial cultures. Bacteriophages have been found for many of the bacterial strains used in the industry, such as species of lactic acid bacteria e.g. *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., and *Streptococcus* spp., *Propionibacterium* spp.,

20 *Bifidobacterium* spp, *Staphylococcus* spp. or *Micrococcus* spp. Furthermore, bacteriophage infections are also well known in other industrially useful species such as *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. *Actinomycetes* spp. and *Brevibacterium* spp.

25 In the food industry lactic acid bacterial starter cultures are widely used for food fermentations. It appears that among members of the lactic acid bacteria *Lactococcus* spp. are most devastated by bacteriophage infections. A factor, which leads to frequent bacteriophage infections in lactic acid bacterial starter cultures is the fact that the fermentation conditions in the food industry including the dairy industry are

30 generally non-sterile. Thus, it has not yet been possible to eliminate bacteriophage contamination under these industrial conditions.

The lytic development of phages involves adsorption of the phages to the host cell surface, injection of phage DNA into the cell, synthesis of phage proteins, replication

of phage DNA, assembly of progeny phages and release of progeny from the host. Cell-mediated mechanisms of interference with any of these events can prevent a phage infection. The ability of bacterial cultures to resist bacteriophage infection during industrial use depends to a large extent on host strain characteristics affecting

5 one or more of the above mechanisms.

Thus, it has been shown that natural bacteriophage resistance or defense mechanisms exist in bacterial strains which ensure a certain level of protection against bacteriophage attack. These natural defense mechanisms include phage adsorption

10 inhibition, prevention of phage DNA penetration, restriction of phage DNA and abortive infection.

The prevention of productive contact between phages and bacterial cells due to altered cell surface receptors for phages greatly reduces the ability of the phages to

15 attack the cells. Adsorption of the phages to the cell surface is not always sufficient for the translocation of the phage DNA. It has been shown that host specific cell membrane proteins are involved in the prevention of phage DNA penetration.

Restriction/modification is a mechanism that operates by the cooperation of two

20 enzyme systems, a DNA-cleaving restriction enzyme and a DNA-modifying enzyme, usually a methylase. The mechanism functions by cleaving the phage DNA, as it enters the cell.

Abortive phage infection is described as a mechanism that interferes with the phage

25 development after phage expression has begun. This may eventually lead to a reduced level or termination of the production of viable phage progeny.

However, like many other traits of bacterial strains which are important for industrial performance, the above described natural phage defense mechanisms have been

30 shown to be unstable characteristics, as they may be mediated by plasmids. Furthermore, these defense mechanisms are often phage specific, i.e. they are only active against a limited range of bacteriophage types. Accordingly, the prior art is not aware of a general and stably maintained host cell associated resistance mechanism against bacteriophage infection.

Based on the above natural defense mechanisms, the industry has designed and implemented strategies to possibly reduce bacteriophage infection of bacterial cultures including starter cultures for the fermentation of dairy products. Currently used
5 strategies include the use of mixed starter cultures and alternate use of strains having different phage susceptibility profiles (strain rotation).

Traditionally, starter cultures in the dairy industry are mixtures of lactic acid bacterial strains. The complex composition of mixed starter cultures ensures that a certain level
10 of resistance to phage attack is present. However, repeated subculturing of mixed strain cultures leads to unpredictable changes in the distribution of individual strains and eventually undesired strain dominance. This in turn may lead to increased susceptibility to phage attack and risk of fermentation failures.

15 Rotation of selected bacterial strains which are sensitive to different phages is another approach to limit phage development. However, it is difficult and cumbersome to identify and select a sufficient number of strains having different phage type profiles to provide an efficient and reliable rotation program. In addition, the continuous use of strains requires careful monitoring for new infectious phages and the need to quickly
20 substitute a strain which is infected by the new bacteriophage by a phage resistant strain. In manufacturing plants where large quantities of bulk starter cultures are made ahead of time, such a quick response is usually not possible.

Studies have shown that a reduced growth capacity of a bacterial culture such as a
25 proteinase-deficient lactic acid bacterium results in reduced phage proliferation (Richardson et al., 1983, 1984). However, such bacterial strains are still growing and are thus still susceptible to attack by phages.

Thus, the industry is not in the possession of any reliable strategy to secure that
30 bacterial cultures used for industrial manufacturing of food products or other products are resistant against attack by bacteriophages. Furthermore, none of the currently used strategies prevent infections by any type of bacteriophages and none of these strategies are capable of precluding that bacteriophages, by a mutational event, circumvent the resistance mechanisms of the bacterial culture strains.

It is therefore a significant objective of the present invention to provide a method of
preventing bacteriophage infection of bacterial cultures which are used in the
manufacturing of food products and other products, wherein the cultures are
5   completely resistant to attack by all types of bacteriophages.

SUMMARY OF THE INVENTION

10   Accordingly, the invention provides in a first aspect a method of modifying a substrate
material by means of a bacterial culture which is capable of being metabolically active
in said substrate, whereby the bacterial culture is not susceptible to attack by
bacteriophages, the method comprising

15   (i) isolating a bacterial strain which is not capable of DNA replication, RNA
transcription or protein synthesis in said substrate material but is capable of
metabolically modifying the substrate material,

(ii) propagating the selected strain in a medium wherein the strain is capable of
20   replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping
the material under conditions where the culture is metabolically active,

25   whereby, if the substrate material is contaminated with a bacteriophage, the metabolic
activity of the bacterial culture is substantially unaffected by the bacteriophage.

The invention pertains in another aspect to a modified lactic acid bacterium that is
modified to become incapable of performing DNA replication, RNA transcription or
30   protein synthesis in a specifically defined substrate material which is limited with
respect to at least one compound that is required by the bacterial strain for DNA
replication, RNA transcription or protein synthesis, said modified bacterial strain is
capable of being metabolically active in said substrate material, whereby the strain is

not susceptible to attack by bacteriophages, subject to the limitation, that the lactic
acid bacterium is not strain DN105 (DSM12289).

In further aspects, there is provided a starter culture compostion comprising the lactic
5 acid bacterium according to the invention and a starter culture compostion comprising
a lactic acid bacterium obtainable by the method according to the invention in
combination with at least one further lactic acid bacterium.

In a further aspect, there is provided a method of manufacturing a food or feed
10 product comprising adding a starter culture composition according to the invention to
a food or feed product starting material and keeping the thus inoculated starting
material under conditions where the lactic acid bacterium is metabolically active.

The invention relates in a further aspect to a method of preventing that a lactic acid
15 bacterial starter culture is infected by bacteriophages in the manufacturing of a food
or feed product, the method comprising adding as a starter culture a lactic acid
bacterium obtained by the method according to the invention to a food or feed
product starting material which is limited with respect to at least one compound that
is required by the bacterial strain for DNA replication, RNA transcription or protein
20 synthesis and keeping the thus inoculated starting material under conditions where the
lactic acid bacterium is metabolically active, whereby, if the substrate material is
contaminated with a bacteriophage, the metabolic activity of the bacterial culture is
substantially unaffected by the bacteriophage.

25 In a still further aspect, the invention pertains to the use of a culture as obtained in
the method of the invention or a lactic acid bacterium according to the invention as a
starter culture in the preparation of a product selected from the group consisting of a
dairy flavour, a product for cheese flavouring, a food product and a feed product.

30

DETAILED DISCLOSURE OF THE INVENTION

Thus, in its broadest aspect the invention provides a method of modifying a substrate
material by means of a bacterial culture which is capable of being metabolically active

in said substrate, whereby the bacterial culture is not susceptible to attack by bacteriophages, said method comprising the steps (i) to (iii) as mentioned above. As used herein, the expression "modifying a substrate material" is used interchangeably with the term "fermentation" and relates to any aerobic or anaerobic breakdown of

5   organic compounds by a bacterial culture with the production of an end product. In addition, it will be appreciated that the expression "metabolically active" refers to the capability of the bacterial culture to convert a substrate material such as e.g. milk or a sugar.

10 In the present context, the expression "not susceptible to attack by bacteriophages" includes the capability of a host cell to be metabolically active even though a bacteriophage adsorbs to the host cell surface and injects its DNA into the host cell. As used herein, the term "bacteriophages" referes to any kind of virus that infects bacteria, including the group of prolate headed bacteriophages, isometric headed

15 bacteriophages and group P335 of bacteriophages.

In accordance with the invention, the method comprises in one aspect the isolation of a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material, but is capable of metabolically

20 modifying said material. It will be understood that in this context the expression "a specifically defined substrate material" referes to substrate material which is limited with respect to at least one nutrient compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis. Evidently, it was a very surprising finding that it is possible to provide such non-proliferating bacterial strains

25 which are unable to grow in specifically defined substrate materials, but which have retained their capability of being metabolically active. As used herein, the expression "non-proliferating bacterial strain" relates to a bacterial strain which is incapable of multiplying in a specifically defined substrate material.

30 In a particularly useful embodiment of the present invention the above specific substrate material is limited with respect to at least one nutrient compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis. Such compounds include amino acids or nitrogenous bases such as purine and pyrimidine bases.

Thus, the growth of the bacterial strain is prevented due to the lack of capability of
the strains to synthesize the specific compound with respect to which the substrate is
limited. Such a mutant strain which has lost the capability of *de novo* synthesising

5 such essentially compounds is also referred to in the art as an "auxotrophic strain".
Therefore, in preferred embodiments, the bacterial strain is a mutant strain being
auxothrophic in respect of a compound which is not present in the substrate material
and which is required by the strain for DNA replication, RNA transcription or protein
synthesis.

10

The substrate material used in the method of the invention may in a further useful
embodiment contain at least one compound that inhibits the DNA replication, RNA
transcription or the protein synthesis of the bacterial strain. Examples of such
compounds include chloramphenicol and erythromycin which affect the ribosomes of

15 the bacterial cell and thus inhibit protein synthesis.

In accordance with the invention, the propagation of the selected strain prior to its use
in the present method requires a medium wherein the strain is capable of replicating to
obtain a culture of said strain. It is assumed that a medium containing the specific

20 compound which the mutant is unable to synthesize, will restore the capability of the
mutant to grow, i.e. capability of DNA replication, RNA transcription and/or protein
synthesis.

In a further step of the method according to the invention, the above obtained

25 bacterial culture is added to the above substrate material and kept under conditions
where the culture is metabolically active. It will be understood that in this context, the
term "conditions" includes the temperature, pH, appropriate composition of the
substrate material or presence/absence of an inducer substance, at which the meta-
bolic activity of the bacterial culture is optimal.

30

Bacteriophages require hosts with intact DNA replication, RNA transcription and
protein synthesis in order to become proliferated. Accordingly, bacterial cultures used
in the method of the invention are incapable of performing one of the above activities,
which makes such bacterial cultures substantially completely resistant to attack by

bacteriophages. Thus, the metabolic activity of the bacterial culture is substantially unaffected even if the substrate material is contaminated with bacteriophages. As used herein, the expression "substantially unaffected" indicates that by using conventional detection methods no changes or only minor changes in the metabolic

5   activity can be detected.

It will be appreciated that such auxotrophic bacteria can be provided by subjecting a wild type bacterial strain that, under appropriate conditions, is capable of growing in a substrate material with or without a specific compound needed for DNA replication,

10 RNA transcription or protein synthesis to a mutagenization treatment and selecting a strain that is substantially incapable of growing in the absence of said specific compound.

Suitable mutagens include conventional chemical mutagens and UV light. Thus, as

15 examples, a chemical mutagen can be selected from (i) a mutagen that associates with or becomes incorporated into DNA such as a base analogue, e.g. 2-aminopurine or an interchelating agent such as ICR-191, (ii) a mutagen that reacts with the DNA including alkylating agents such as nitrosoguanidine or hydroxylamine, or ethane methyl sulphonate (EMS).

20

As an alternative, auxotrophic bacteria can be provided by selecting spontaneously occurring mutants which, compared to the parent strain, has a growth requirement for a compound needed for DNA replication, RNA transcription or protein synthesis.

25 It will be understood that it is also possible to provide an auxotrophic mutant by site-directed mutagenesis, e.g. by using recombinant DNA techniques, such as gene knock-out techniques, by which the specific gene is disrupted and rendered non-functional in the bacterium. It is also possible to construct the mutated bacterial strains according to the method of the present invention by techniques which involve

30 the loss of part of the chromosome or a nucleotide base or bases in the DNA sequence which renders the specific gene non-functional in the bacterium. An illustrative example of such a deletion strategy is described in details in the below Examples.

The above bacterial wild type parent strain can be selected from any industrially suitable bacterial species, i.e. the strain can be selected from the group consisting of *Lactococcus* spp. including *L. lactis*, *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp.,

5 *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Actinomycetes* spp., *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. and *Brevibacterium* spp.

In one specific embodiment of the method according to the invention, a Pur⁻ mutant,

10 including the *Lactococcus lactis* strain DN105 (DSM 12289), is used. This acidifying bacterial strain DN105 is a purine auxotrophic mutant capable of acidifying milk, even though the strain is not capable of growth because of its requirement for purine, which is not present in milk in sufficient amounts to support growth of such a bacterium. As it is shown in the below Example 1, strain DN105 is capable of

15 acidifying milk under purine starvation conditions even in the presence of a high concentration of bacteriophages.

In a further useful embodiment of the method according to the invention, a *thyA* mutant, including the *Lactococcus lactis* strain MBP71 (DSM12891), is used. This

20 acidifying bacterial strain MBP71 is a thymidine auxotrophic mutant, which is capable of acidifying milk, even though the strain is not capable of growth, due to its requirement for thymidine, which is not present in milk in sufficient amounts to support growth of such a mutant. As shown in Example 2 below, strain MBP71 is capable of acidifying milk under thymidine starvation conditions even in the presence

25 of a high concentration of bacteriophages.

It is convenient to provide the above bacterial strains both when used as a food or feed production strain and as a production strain for metabolites, as a composition comprising the bacterial strain selected for the specific use. Typically, such

30 compositions contain the bacterium in concentrated form e.g. at a concentration of viable cells (colony forming units, CFUs) which is in the range of 10^5 to 10^{13} per g of the composition such as in the range of 10^8 to 10^{12} per g. Additionally, the culture composition may contain further components such as bacterial nutrients, cryoprotec-tants or other substances enhancing the viability of the bacterial active ingredient

10

during storage. The composition can e.g. be in the form of a liquid, frozen or a freeze-
dried composition.

As mentioned above, one characteristic of the bacterial culture as used in the method
5   of the invention, is that it is capable of metabolically modifying a specifically defined
substrate material even though the strain is incapable of growth in such substrate.
When a lactic acid bacterium is used in the method of the invention, the obtained
bacterial culture typically has an acidification rate in milk which is at least 10% of that
of said culture when it is present in a substrate material where it is capable of DNA
10   replication, RNA transcription and/or protein synthesis. In preferred embodiments, the
bacterial strain has an acidification rate in milk which is at least 1% including at least
5%, such as at least 10%, e.g. at least 15%, such as at least 20%, including at least
25% of that of the culture when it is present in a substrate material where it is
capable of DNA replication, RNA transcription and/or protein synthesis.
15

Typically, the bacterial strain used in the method of the invention is added to the
substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the
material, such as at least 10^5 CFU/ml or g of the material, including at least 10^6
CFU/ml or g of the material, such as at least 10^7 CFU/ml or g of the material, e.g. at
20   least 10^8 CFU/ml or g of the material, including at least 10^9 CFU/ml or g of the
material.

Thus, the obtainment of a bacterial culture which is completely resistant to
bacteriophage attack according to the method of the invention, can be utilized in all
25   industrial contexts where proliferation of the culture in the substrate material is not a
requirement. Dairy fermentations of milk is such an example of an industrial
manufacturing process, as proliferation of the lactic acid bacteria during milk fermen-
tation is not a requirement, if the desired taste and acidification of the fermentation
product are obtained. Therefore, in useful embodiments, the substrate material is a
30   starting material for an edible product including milk, a vegetable material, a meat
product, a must, a fruit juice, a wine, a dough and a batter.

In further embodiments, the substrate material is a starting material for an animal feed
such as silage e.g. grass, cereal material, peas, alfalfa or sugar-beet leaf, where

bacterial cultures are inoculated in the feed crop to be ensiled in order to obtain a preservation hereof, or in protein rich animal waste products such as slaughtering offal and fish offal, also with the aims of preserving this offal for animal feeding purposes.

5 Yet another significant application of the method according to the present invention is the use of the bacterial cultures as so-called probiotics. By the term "probiotic" is in the present context understood a microbial culture which, when ingested in the form of viable cells by humans or animals, confers an improved health condition, e.g. by suppressing harmful microorganisms in the gastrointestinal tract, by enhancing the
10 immune system or by contributing to the digestion of nutrients.

It is, as mentioned above, an important objective of the present invention to provide a method of preventing bacteriophage infection of bacterial cultures which are metabolically active by using non-proliferating bacterial cells. In order to be of
15 industrial interest, such metabolic activity should result in the production of a substantial amount of the desired end product. Thus, one possibility of increasing such production by use of a non-proliferating cell is an enhancement of the flux through metabolic pathways.

20 Accordingly, in one useful embodiment, the bacterial culture used in the method of the invention comprises a genetically modified strain which, relative to its parent strain, is enhanced in at least one metabolic pathway. Such enhanced metabolic activity can e.g. be obtained through an enhanced glycolytic pathway and/or an enhanced flux through the pentose phosphate pathway.
25

One approach for stimulating the flux through the glycolytic pathway is by increasing the expression of ATPase activity, i.e. an enhanced conversion of ATP to ADP, as described in WO 98/10089. Thus, in one useful embodiment of the invention, the genetically modified strain has, relative to its parent strain, an enhanced ATPase
30 activity.

In one interesting embodiment of the present invention, the genetically modified strain is one wherein the gene coding for an ATPase is under the control of a regulatable promoter. As used herein, the term "regulatable promoter" is used to describe a

promoter sequence possibly including regulatory sequences for the promoter, which promoter is regulatable by one or more factors present in the environment of the strain. Such factors include the pH of the growth medium, the growth temperature, a temperature shift eliciting the expression of heat shock genes, the composition of the

5   growth medium including the ionic strength/NaCl content and the growth phase/growth rate of the bacterium. Such a regulatable promoter may be the native promoter or it may be an inserted promoter not naturally related to the gene either isolated from the same bacterial species or it may be a heterologous promoter sequence, i.e. a sequence derived from different bacterial species.

10

Cells such as "resting cells" or "non-dividing cells" represent other types of non-proliferating cells which are useful in the method of the invention and wherein the above enhancement of the flux through the metabolic pathways is useful. Such cells are incapable of mitosis or meiosis e.g. due to the deficiency of DNA, RNA and/or

15 protein needed for the separation of the cell. Thus, in a particularly useful embod-iment, the bacterial culture is one which comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

In addition, the invention encompasses non-proliferating strains which under specific

20 conditions are incapable of growth. Thus, in an interesting embodiment, the bacterial culture comprises a strain which is a conditional mutant, i.e. a mutant which under predetermined conditions does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis. Such predetermined conditions include pH, temperature, composition of the substrate

25 material and presence/absence of inducer substances.

One possible means of providing such conditional mutants which are temperature-sensitive is by subjecting a bacterial strain that under appropriate conditions is capable of growing in a substrate material e.g. at a temperature below 30°C to a

30 mutagenization treatment and selecting a mutant strain that is substantially incapable of growth at temperatures below 30°C, but is capable of growth at higher temperatures.

In a further aspect of the invention there is provided a modified lactic acid bacterium that is modified to become incapable of performing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA
5   replication, RNA transcription or protein synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

10   In useful embodiments, the lactic acid bacterium according to the invention is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

Presently preferred lactic acid bacteria according to the invention are mutant strains
15   which are *thyA* mutants including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

The modified lactic acid bacterium according to the invention is useful as a starter culture in the production of food and feed products. Accordingly, in a further
20   important aspect the invention relates to a starter culture composition comprising the lactic acid bacterium according to the invention.

As it is normal in the production of lactic acid bacterial fermentation processes to apply mixed cultures lactic acid bacteria, a composition will in certain embodiments
25   comprise a multiplicity of strains either belonging to the same species or belonging to different species. Accordingly, in a further important aspect, the invention relates to a starter culture composition comprising a lactic acid bacterium obtainable by the method according to the invention in combination with at least one further lactic acid bacterium. A typical example of such a useful combination of lactic acid bacteria in a
30   starter culture composition is a mixture of the bacterium obtainable by the method according to the invention and one or more *Lactococcus* spp. such as *Lactococcus lactis* subsp. *lactis* or *Lactococcus lactis* subsp. *lactis* biovar. *diacetylactis* or *Leuconostoc* spp. Such a mixed culture can be used in the manufacturing of fermented milk products such as buttermilk and cheese.

In one embodiment, the composition according to the invention, is one which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

5

It is also an objective of the present invention to provide a method of manufacturing a food or feed product based on the use of the modified bacterium of the invention. Thus, in its broadest aspect, such a method comprises adding a starter culture composition according to the invention to a food or feed product starting material and

10 keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active. In a particularly useful embodiment, the food product starting material is milk.

In further aspects, the invention relates to the use of a culture as obtained in the

15 method according to the invention as a starter culture in the preparation of a product selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

The invention will now be described in further details in the following non-limiting

20 examples and the drawings wherein

Fig. 1. shows the acidification of reconstituted skim milk (RSM) by strain DN105. The pH was followed over time in RSM cultures containing strain *Lactococcus lactis* DN105 inoculated at 1%, 10%, 25% and 50% vol/vol;

25

Fig. 2 illustrates the development of pH in RSM when inoculated with 25% vol/vol of *Lactococcus lactis* strain CHCC373 or *Lactococcus lactis* strain DN105, in the presence of the bacteriophage strain 836 at a concentration of at least 10^8 PFU/ml;

30 Fig. 3A-3H illustrate the development of pH in RSM when inoculated with 25% vol/vol of *Lactococcus lactis* strain DN105 with or without addition of the purine compound hypoxanthine to the culture medium, in the presence of at least 10^8 PFU/ml of the following bacteriophages: CHPC836 (3A); CHPC412 (3B); CHPC783 (3C); CHPC795 (3D); CHPC710 (3E); CHPC12 (3F); CHPC708 (3G); CHPC814 (3H);

Fig. 4. shows the acidification of RSM by *Lactococcus lactis* strain MBP71 inoculated
at different concentrations. The pH of 200 ml RSM cultures comprising strain MBP71
inoculated at 1%, 5%, 10%, 25%, and 50% (v/v) was monitored on-line; and

5

Fig. 5. shows the acidification of RSM, comprising bacteriophages and/or thymidine,
by *Lactococcus lactis* strains MBP71 and CHCC373, which were inoculated at a
concentration of 25% (v/v) in a volume of 100 ml RSM. The thymidine concentration
was 20 mg/l and bacteriophage CHPC733 was added to a concentration of $2x10^8$
10 PFU/ml. The pH was monitored by collecting 2.5 ml samples.

EXAMPLE 1

15 The use of a purine auxotrophic *Lactococcus lactis* strain for obtaining resistance
against bacteriophages in milk fermentations

1.1. Materials and methods

20 (i) Bacterial strains, media and growth conditions

The *Lactococcus lactis* strain DN105 is a purine auxotrophic mutant (Pur⁻) derived
from the wild type strain CHCC373 described in Nilsson and Lauridsen (1992). A
sample of *Lactococcus lactis* strain DN105 is deposited with Deutsche Sammlung von
25 Mikroorganismen und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124
Braunschweig, Germany on 30 June 1998 under the accession No. DSM 12289.

Lactococcus lactis was grown in M17 (Terzaghi & Sandine, 1975) in the chemically
defined DN medium (Dickely et al., 1995) or in reconstituted skim milk (RSM)
30 containing 9.5% (w/vol) low fat skim milk powder at 30°C. For the propagation of
strain DN105, the medium was supplemented with hypoxanthine to a final
concentration of 15-50 mg/l. The Pur⁻ phenotype of strain DN105 was tested by its
ability to grow on DN medium with and without hypoxanthine supplement.

(ii) Bacteriophages and their handling

Bacteriophages were purified by three single plaque isolation steps. The high titer bacteriophage lysates were prepared by consecutive infections of the host strain

5 CHCC373 with the bacteriophage at an MOI (multiplicity of infection) of 0.1 to 1.0. After infection the culture was grown at 30°C in M17 supplemented with 10 mM CaCl$_2$ until completed lysis. The lysates were centrifuged for 15 min at 6.000 rpm and the supernatant sterile filtered (0.45 μm, Schleicher und Schuell).

10 (iii) Determination of bacteriophage titers

For the determination of bacteriophage titers (plaque forming units per ml) the agar double layer method was used (Adams M.H., 1959; Interscience Publishers, Inc., New York). The bacteriophages used for the acidification test are listed in Table 1 below:

15

Table 1. Bacteriophages and phage titers

Phage	Titer (PFU/ml)
CHPC12	4×10^{10}
CHPC412	3×10^{10}
CHPC708	2×10^{10}
CHPC710	2×10^{10}
CHPC783	1×10^{11}
CHPC795	7×10^{10}
CHPC814	1×10^{10}
CHPC836	4×10^{10}

20 (iv) Test for the acidification of RSM by strain DN105

10-400 ml of an outgrown culture of strain DN105 in M17 was harvested by centrifugation, washed twice with a sterile solution of 0.9% NaCl to remove residual purine compounds and resuspended in 10-400 ml of RSM to give the same cell

density as in the outgrown M17 culture. The resuspended material was used for inoculation of fresh RSM at volume/volume concentrations typically in the range of 10 to 100% (v/v). The pH was monitored either on-line or by measuring the pH of 3 ml samples collected at intervals.

5

<u>1.2. Results of the acidification of RSM by *Lactococcus lactis* strain DN105</u>

In general, lactic acid bacterial cells which do not have intact DNA replication, RNA transcription and protein synthesis systems are unable to grow and acidify milk.

10 Nilsson and Lauridsen (1992) demonstrated that the purine auxotrophic mutant DN105 is unable to grow in a medium without purines. It has also been reported that purine auxotrophic mutants of *Lactococcus lactis* not growing in milk are incapable of acidifying such a substrate material (Dickely et al., 1995). To test the ability of strain DN105 to acidify a milk based medium, the strain was inoculated in varying amounts

15 in RSM as described in Materials and Methods and the pH of the substrate material was monitored (Fig. 1).

The results shown in Fig. 1 clearly demonstrates that strain DN105 was able to acidify milk at least to pH 5.0 even under purine starving conditions.

20

<u>1.3 Studies of the bacteriophage resistance of *Lactococcus lactis* strain DN105 in RSM</u>

As it is generally known, a bacteriophage requires a susceptible host cell which has

25 intact DNA replication, RNA transcription and protein synthesis systems for development. If a potential host is not capable of performing at least one of these activities, bacteriophages cannot be proliferated in the cell.

Fig. 2 shows the development of pH in RSM when inoculated with 25% vol/vol of the

30 wild type strain CHCC373 or the mutant strain DN105 in the presence of the bacteriophage CHPC836 at a concentration of at least 10^8 PFU/ml.

In further experiments the resistance of strain DN105 against various bacteriophages was studied in RSM with and without hypoxanthine added. The bacteriophages were added to RSM at 10^8 to 10^9 bacteriophages/ml.

5 The addition of hypoxanthine was used as a positive control for the bacteriophage infection, as the addition of this purine compound enabled bacteriophage attack (Fig. 3 A-H). In all cultures without hypoxanthine added strain DN105 acidified the milk to around pH 5.0, whereas with the addition of hypoxanthine none of the cultures reached a pH of below 5.4.

10

1.4 Discussion

This Example shows that the starvation of a Pur⁻ strain of *L. lactis* for purines causes total resistance to a range of bacteriophages and that the Pur⁻ strain of *L. lactis* is

15 capable of effectively acidifying milk in the presence of a large number of bacteriophages for which the corresponding wild type is susceptible.

Thus, the present Example shows that it is possible to develop strains which under appropriate selected conditions, where the strains are incapable of growth, are

20 completely resistant to bacteriophages and that such strains have retained the metabolic ability to acidify milk. Such a system of bacteriophage resistance can be introduced into any bacteria e.g. *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp., *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Actinomycetes* spp.,

25 *Enterobacteriaceae* spp. including *E. coli*, *Corynebacterium* spp. and *Brevibacterium* spp. in regard to e.g. production of a bacterial fermentation product such as lactate, diacetyl, acetoin, methanethiol, ethanol etc. if proliferation of the bacteria is not a re-quirement.

30

EXAMPLE 2

The use of a thymidine auxotrophic *Lactococcus lactis* strain for obtaining resistance against bacteriophages in milk fermentations

2.1. Materials and methods

(i) Bacterial strains, bacteriophages, media and growth conditions

5

The *Lactococcus lactis* strain MBP71 is a thymidine auxotrophic mutant (*thyA*) derived from the wild type strain CHCC373 (see Example 1). A sample of strain MBP71 has been deposited with Deutsche Sammlung von Mikroorganism und Zellkulturen GmbH, Mascheroder Weg 1b, D-38124 Braunschweig, Germany on 25 June 1999 under the

10   accession No. DSM12891.

Strain CHCC373 was routinely grown at 30°C in M17 (Terzaghi & Sandine, 1975) supplemented with 0.5% of lactose, or in reconstituted skim milk (RSM) containing 9.5% (w/v) low fat skim milk powder. For the propagation of strain MBP71 the media

15   were supplemented with 20 mg/l of thymidine, since neither M17 nor RSM support the growth of the ThyA⁻ phenotype. During the manipulations to construct strain MBP71 5 mg/l of erythromycin was added to the M17 based media to maintain the plasmid. Also, when growth at 37°C was required during these manipulations M17 based media were supplemented with 50 mM of NaCl to enhance growth. *E. coli*

20   DH5α (Life Technologies Inc., USA) was grown in LB medium supplemented with 100 mg/l of erythromycin to maintain the transformed plasmid. The bacteriophage CHPC733 was used in growth experiments where strain MBP71 phage resistance was tested. For the bacteriophage handling and determination of titers see Example 1.

25   (ii) Construction of plasmid with deletion in *thyA* from strain CHCC373

An about 800 bp fragment upstream of *thyA* from strain CHCC373 was obtained by PCR on chromosomal DNA using the primers

30   TATAATCTGCAGGGTCACACTATCAGTAATTG (SEQ ID NO:1) and
        TATTTTAAGCTTCACAGTCTGCTATTTTGATTC (SEQ ID NO:2),

which furthermore introduce a *Pst*I and a *Hind*III site, respectively, in the fragment ends. The resulting fragment includes the -35 box of the *thyA* promoter, but not the -

10 box. Another about 800 bp fragment comprising an internal part of the *thyA* coding region was obtained by PCR on chromosomal DNA using the primers

TAAATTAAGCTTCGCAGACAAGATTTTTAAAC (SEQ ID NO:3) and

5   ATTTAAGTCGACGGCTCATAGTCCACAAGTTC (SEQ ID NO:4),

which introduce a *Hind*III and a *Sal*I site, respectively. The resulting fragment include the whole coding region of *thyA* except the first 8 bp and the last 34 bp. The two PCR fragments were purified by using the QIAquick PCR purification kit (QIAGEN

10 GmbH, Germany), cut with the indicated enzymes, and purified again. The pGhost9 vector (Maguin et al., 1996) was cut with the restriction enzymes *Pst*I and *Sal*I and purified. Thereafter, about 150 ng of the vector and about 50 ng of each of the two fragments were ligated overnight at 16°C in a total volume of 20μl. From this mixture 10 μl was used to transform *E. coli* DH5α. Plasmid DNA was isolated from possible

15 clones by growing them overnight in LB medium with 100 mg/l of erythromycin and subsequently using 1.5 ml of these cultures with the QIAprep spin miniprep kit protocol (QIAGEN GmbH, Germany). The purified plasmid DNA was cut with *Pst*I and *Sal*I and one of them, pMBP63, yielded a band of approx. 1600 bp. This construct was further verified by PCR with the primers that were used to produce the two

20 fragments, and also with the two outer primers. Finally, the construct was verified by sequencing both strands over the region of the deletion with the two primers

GACTGTTGCCCCATAGCG (SEQ ID NO:5) and
GCTTCGATTTTAGTATATGG (SEQ ID NO:6).

25

All primers were from TAG Copenhagen A/S, Copenhagen, Denmark.

(iii) Inactivation of the chromosomal *thyA* gene in strain CHCC373

30 About 200 ng of pMBP63 was used to transform strain CHCC373. By employing the gene replacement feature (Biswas et al., 1993) of the pGhost9 vector the chromosomal *thyA* gene of strain CHCC373 was thereafter successfully inactivated. The resulting strain, MBP71, was shown to be a thymidine auxotrophic mutant.

(iv) Test for acidification of RSM by strains CHCC373 and MBP71

Overnight cultures of M17 with 0.5% lactose and 20 mg/l of thymidine were washed twice in an isotonic solution of the same volume as the overnight culture to remove

5   residual thymidine. The cells were resuspended in a fresh isotonic solution of a volume which was 1/20 of the overnight culture. These resuspended cells were used to inoculate RSM to a given volume identified by the percentage volume of the overnight culture i.e. an inoculation of 100% (v/v) indicates that 5 ml of the resuspended cells have been used to inoculate 100 ml of RSM. The pH was monitored either on-line or

10 by measuring the pH of 2.5 ml samples.

2.2. Results of the acidification of RSM by *Lactococcus lactis* strain MBP71

To test the acidification of RSM by strain MBP71 this strain was inoculated in varying

15 amounts as described in materials and methods. The pH of the substrate material was then monitored on-line over a 20 hour period. The results shown in Fig. 4 clearly demonstrate that MBP71 is able to acidify milk down to at least pH 4.5 under thymidine starvation conditions. When thymidine was added to the RSM, strain MBP71 acidified the substrate material similarly to strain CHCC373 (details not

20 shown).

2.3. Studies of the bacteriophage resistance of *Lactococcus lactis* MBP71 in RSM

As described above, a bacteriophage requires a susceptible host cell, which has intact

25 DNA replication, RNA transcription and protein synthesis systems, for development. If a potential host is not capable of performing just one of these activities, bacteriophages can not proliferate in the cell. The thymidine auxotrophic strain MBP71 is only capable of DNA replication in the presence of thymidine, or another suitable precursor for dTTP. None of these precursors are present in RSM, and strain MBP71 is

30 therefore not susceptible to bacteriophage attack under these conditions.

Fig. 5 shows the development of pH in 100 ml RSM inoculated with strain MBP71 at a concentration of 25% (v/v). When thymidine was present it was added to a final concentration of 20 mg/l. When the bacteriophage CHPC733 was present it was

added to a concentration of 2×10^8 PFU/ml half an hour after cell inoculation. This gives a MOI (multiplicity of infection) of 0.2. The results in Fig. 5 clearly demonstrate that strain MBP71 acidifies RSM without thymidine down to at least pH 4.6 even in the presence of bacteriophages. However, when thymidine is added to the RSM strain

5 MBP71 becomes susceptible to bacteriophage attack and the milk is only acidified down to pH 5.5, which is also close to the pH that is reached for the wild type strain CHCC373 in the presence of bacteriophages. If the bacteriophages are omitted, but thymidine is added, strain MBP71 acidifies the RSM similarly to strain CHCC373 (see Fig. 5, details not shown).

10

2.4. Discussion

This example shows that the starvation of a *thyA* strain of *L. lactis* for thymidine causes total resistance to bacteriophage CHPC733 and that the *thyA* strain of *L. lactis*

15 is capable of effectively acidifying milk under these conditions. This is contrary to the wild type, which is not able to acidify the milk to an acceptable level under the same conditions. Although, strain MBP71 has only been tested for bacteriophage resistance to one bacteriophage it is believed that strain MBP71 is resistant to all, or most, types of bacteriophages.

20

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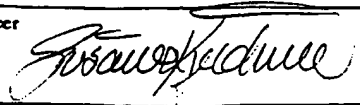
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Applicant's or agent's file reference number	21134 PC 1	International application No.	

INDICATIONS RELATING TO A DEPOSITED MICROORGANISM

(PCT Rule 13bis)

A. The indications made below relate to the microorganism referred to in the description

on page _____15_____ , line _____24_____

B. IDENTIFICATION OF DEPOSIT   Further deposits are identified on an additional sheet [X]

Name of depositary institution

DSMZ-Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH

Address of depositary institution (including postal code and country)

Mascheroder Weg 1B
D-38124 Braunschweig
Germany

Date of deposit 30 June 1998	Accession Number DSM 12289

C. ADDITIONAL INDICATIONS (leave blank if not applicable)   This information is continued on an additional sheet [X]

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganisms only be made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

D. DESIGNATED STATES FOR WHICH INDICATIONS ARE MADE (if the indications are not for all designated States)

E. SEPARATE FURNISHING OF INDICATIONS (leave blank if not applicable)

The indications listed below will be submitted to the International Bureau later (specify the general nature of the indications e.g., "Accession Number of Deposit")

For receiving Office use only	For International Bureau use only
[X] This sheet was received with the international application	[ ] This sheet was received by the International Bureau on:
Authorized officer	Authorized officer

Form PCT/RO/134 (July 1992)

INDICATIONS RELATING TO DEPOSITED MICROORGANISMS
(PCT Rule 12bis)

5   Additional sheet

In addition to the microorganism indicated on page 24 of the description, the following microorganism has been deposited with

10   DSM-Deutsche Sammlung von Mikroorganismen und Cellkulturen GmbH
        Mascheroder Weg 1b, D-38124 Braunschweig, Germany

on the dates and under the accession number as stated below:

15

Accession number	Date of deposit	Description Page No.	Description Line No.
20 DSM 12891	25 June 1999	19	7

For the above-identified deposited microorganism, the following additional indications
25 apply:

As regards the respective Patent Offices of the respective designated states, the applicants request that a sample of the deposited microorganism stated above only be
30 made available to an expert nominated by the requester until the date on which the patent is granted or the date on which the application has been refused or withdrawn or is deemed to be withdrawn.

CLAIMS

1. A method of modifying a substrate material by means of a bacterial culture which is capable of being metabolically active in said substrate, whereby the bacterial culture is
5 not susceptible to attack by bacteriophages, the method comprising

(i) isolating a bacterial strain which is not capable of DNA replication, RNA transcription or protein synthesis in said substrate material but is capable of metabolically modifying the substrate material,
10

(ii) propagating the selected strain in a medium wherein the strain is capable of replicating to obtain a culture of said strain,

(iii) adding the thus obtained bacterial culture to the substrate material and keeping the
15 material under conditions where the culture is metabolically active.

whereby, if the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.

20 2. A method according to claim 1 wherein the substrate material is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis.

3. A method according to claim 2 wherein the bacterial strain is a mutant strain being
25 auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

4. A method according to claim 3 wherein the mutant strain is a Pur⁻ mutant including
Lactococcus lactis strain DN105 deposited under the accession number DSM 12289.
30
5. A method according to claim 3 wherein the mutant strain is a *thyA* mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

6. A method according to any of claims 2 to 5 wherein the strain in said substrate material is not capable of performing at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

5 7. A method according to claim 1 wherein the substrate material contains at least one compound that inhibits the DNA replication, RNA transcription or the protein synthesis of the bacterial strain.

8. A method according to claim 1 wherein the substrate material is a starting material
10 for an edible product, the material is selected from the group consisting of milk, a vegetable material, a meat product, a must, a fruit juice, a wine, a dough and a batter.

9. A method according to claim 1 wherein the bacterial culture is selected from the group consisting of *Lactococcus* spp., *Lactobacillus* spp., *Leuconostoc* spp.,
15 *Pediococcus* spp., *Streptococcus* spp., *Propionibacterium* spp., *Bifidobacterium* spp., *Staphylococcus* spp., *Micrococcus* spp., *Bacillus* spp., *Enterobacteriaceae* spp. including *E. coli, Actinomycetes* spp., *Corynebacterium* spp. and *Brevibacterium* spp.

10. A method according to claim 9 wherein the bacterial culture is of *Lactococcus*
20 *lactis*.

11. A method according to claim 1 wherein the bacterial strain is added to the substrate material at a concentration in the range of 10^5 to 10^9 CFU/ml or g of the material.
25

12. A method according to claim 1 wherein the culture comprises a genetically modified strain which, relative to its parent strain is enhanced in at least one metabolic pathway.

30 13. A method according to claim 12 wherein the genetically modified strain has, relative to its parent strain, an enhanced metabolic activity selected from the group consisting of enhanced glycolytic flux and enhanced flux through the pentose phosphate pathway.

14. A method according to claim 13 wherein the genetically modified strain has, relative to its parent strain, an enhanced ATPase activity.

15. A method according to claim 1 wherein the bacterial culture comprises a strain
5   which is a conditional mutant which at a predetermined condition does not perform at least one activity selected from the group consisting of DNA replication, RNA transcription and protein synthesis.

16. A method according to claim 15 wherein the predetermined condition is selected
10 from the group consisting of pH, temperature, composition of the substrate material and presence/absence of an inducer substance.

17. A method according to claim 1 wherein the culture comprises a bacterial strain which is capable of increasing the size of the cells without mitosis.

15

18. A modified lactic acid bacterium that is modified to become incapable of perform-ing DNA replication, RNA transcription or protein synthesis in a specifically defined substrate material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein
20 synthesis, said modified bacterial strain is capable of being metabolically active in said substrate material, whereby the strain is not susceptible to attack by bacteriophages, subject to the limitation, that the lactic acid bacterium is not strain DN105 (DSM12289).

25 19. A lactic acid bacterium according to claim 18 wherein the bacterial strain is a mutant strain being auxothrophic in respect of a compound which is not present in the substrate material and which is required by the strain for replication.

20. A lactic acid bacterium according to claim 19 wherein the mutant strain is a *thyA*
30 mutant including *Lactococcus lactis* strain MBP71 deposited under the accession number DSM12891.

21. A starter culture compostion comprising the lactic acid bacterium of any of claims 18-20.

22. A starter culture composition comprising a lactic acid bacterium obtainable by the method according to claim 1 in combination with at least one further lactic acid bacterium.

5

23. A composition according to claim 22 which further comprising at least on component enhancing the viability of the bacterial active ingredient during storage including a bacterial nutrient or a cryoprotectant.

10   24. A method of manufacturing a food or feed product comprising adding a starter culture composition according to any of claims 21-23 to a food or feed product starting material and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active.

15   25. A method according to claim 24 wherein the food product starting material is milk.

26. Use of a culture as obtained in the method of claim 1 or a lactic acid bacterium according to any of claims 18-20 as a starter culture in the preparation of a product
20   selected from the group consisting of a dairy flavour, a product for cheese flavouring, a food product and a feed product.

27. A method of preventing that a lactic acid bacterial starter culture is infected by bacteriophages in the manufacturing of a food or feed product, the method comprising
25   adding as a starter culture a lactic acid bacterium obtained by the method according to claim 1 to a food or feed product starting material which is limited with respect to at least one compound that is required by the bacterial strain for DNA replication, RNA transcription or protein synthesis and keeping the thus inoculated starting material under conditions where the lactic acid bacterium is metabolically active, whereby, if
30   the substrate material is contaminated with a bacteriophage, the metabolic activity of the bacterial culture is substantially unaffected by the bacteriophage.
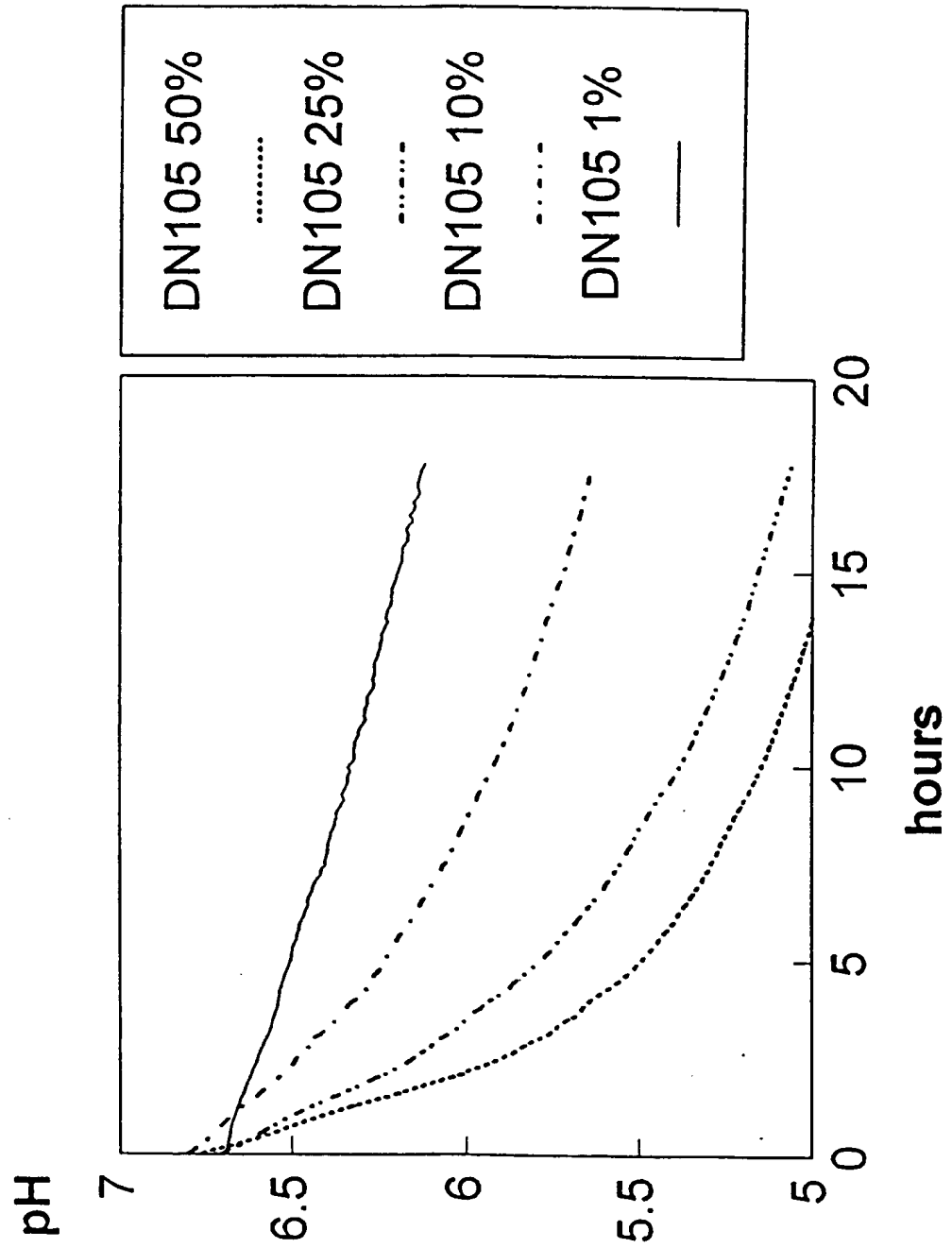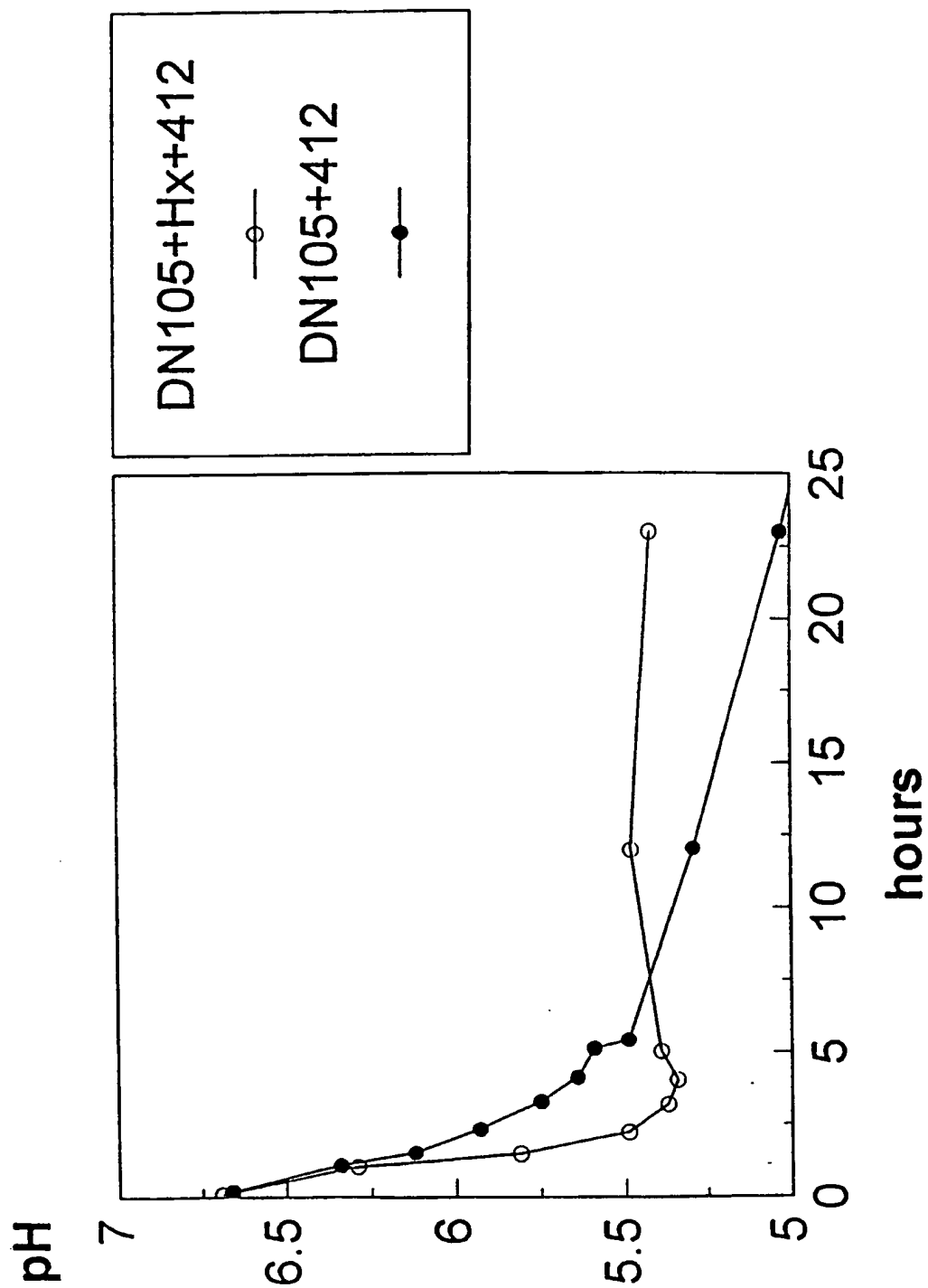
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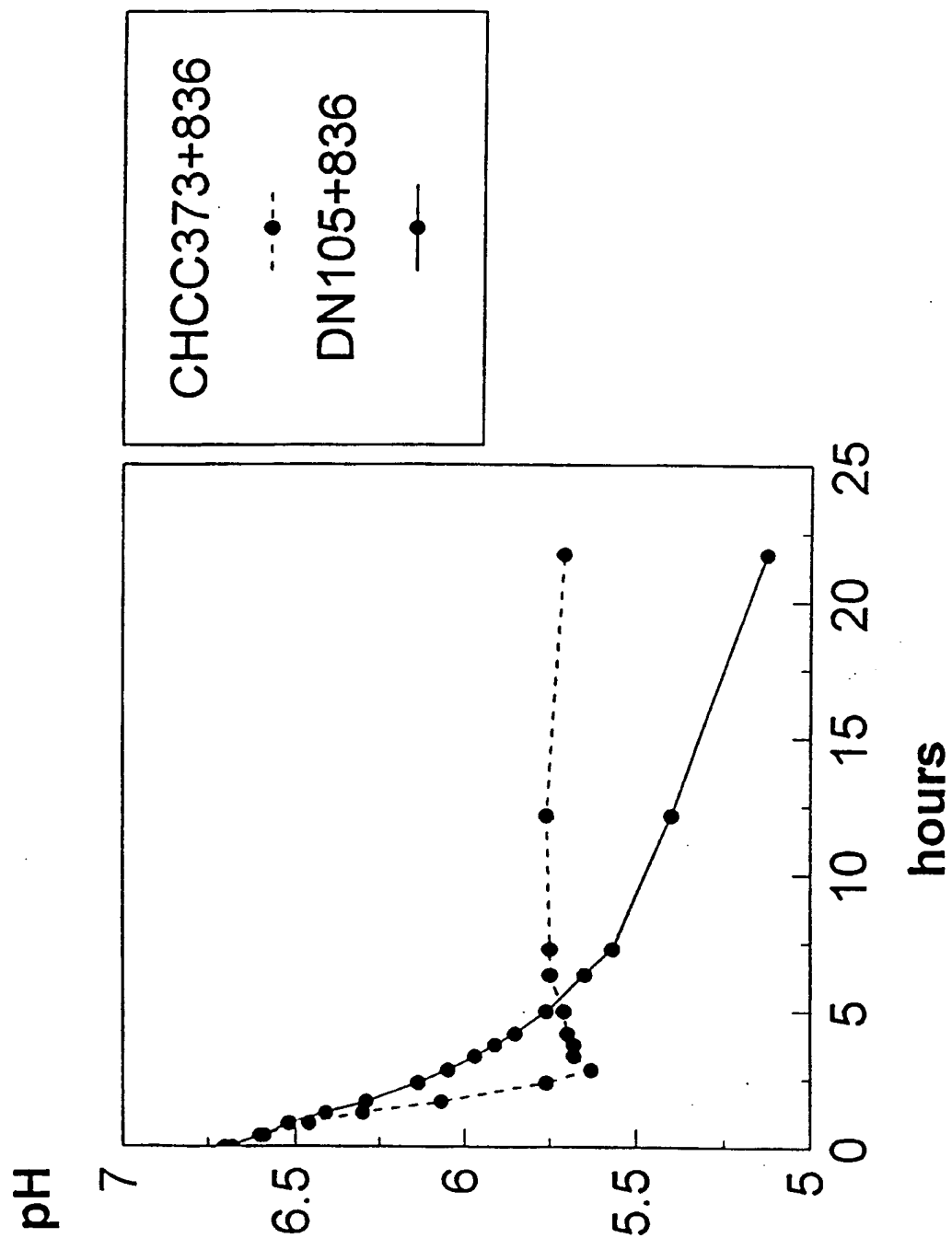
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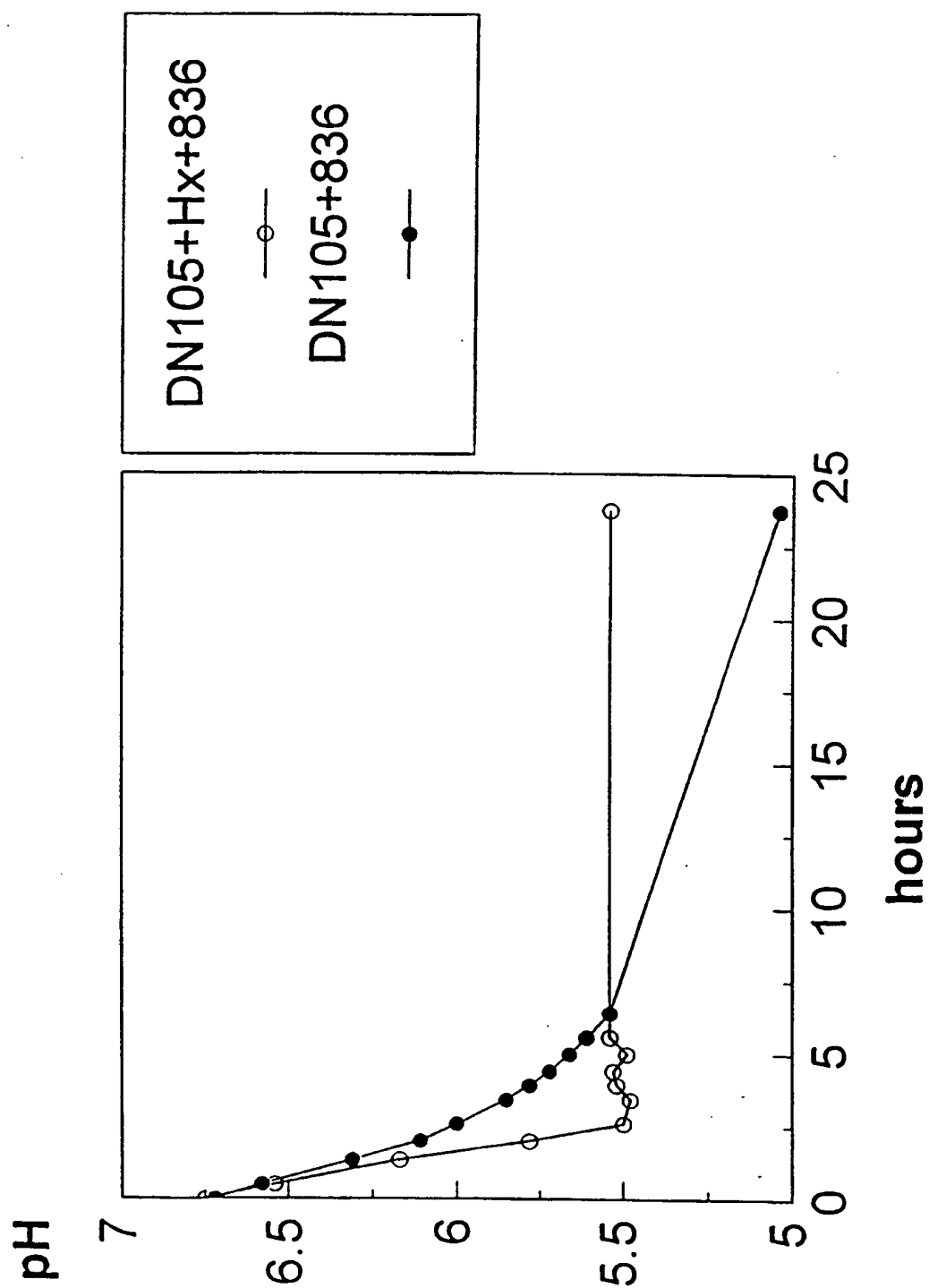
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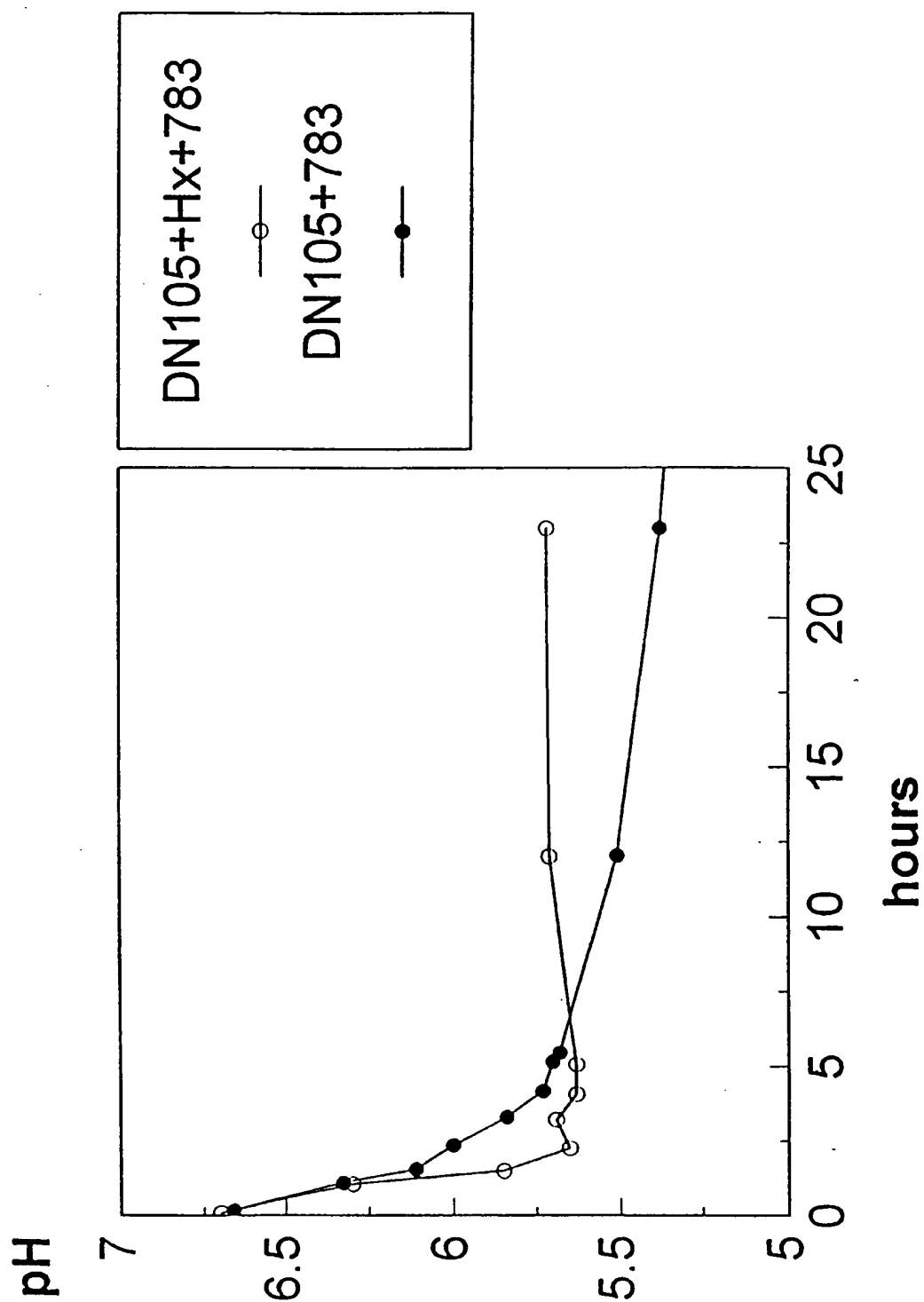
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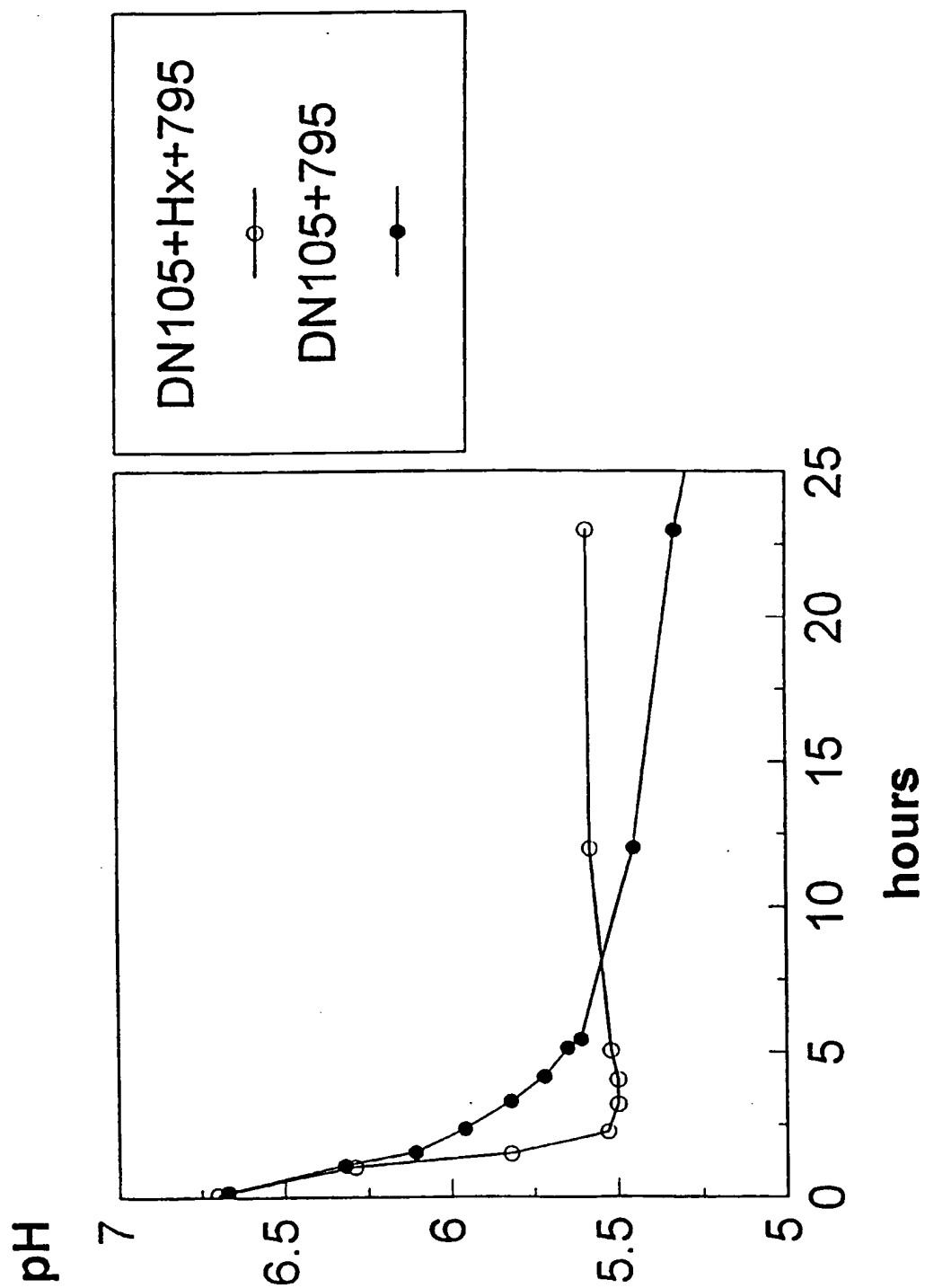
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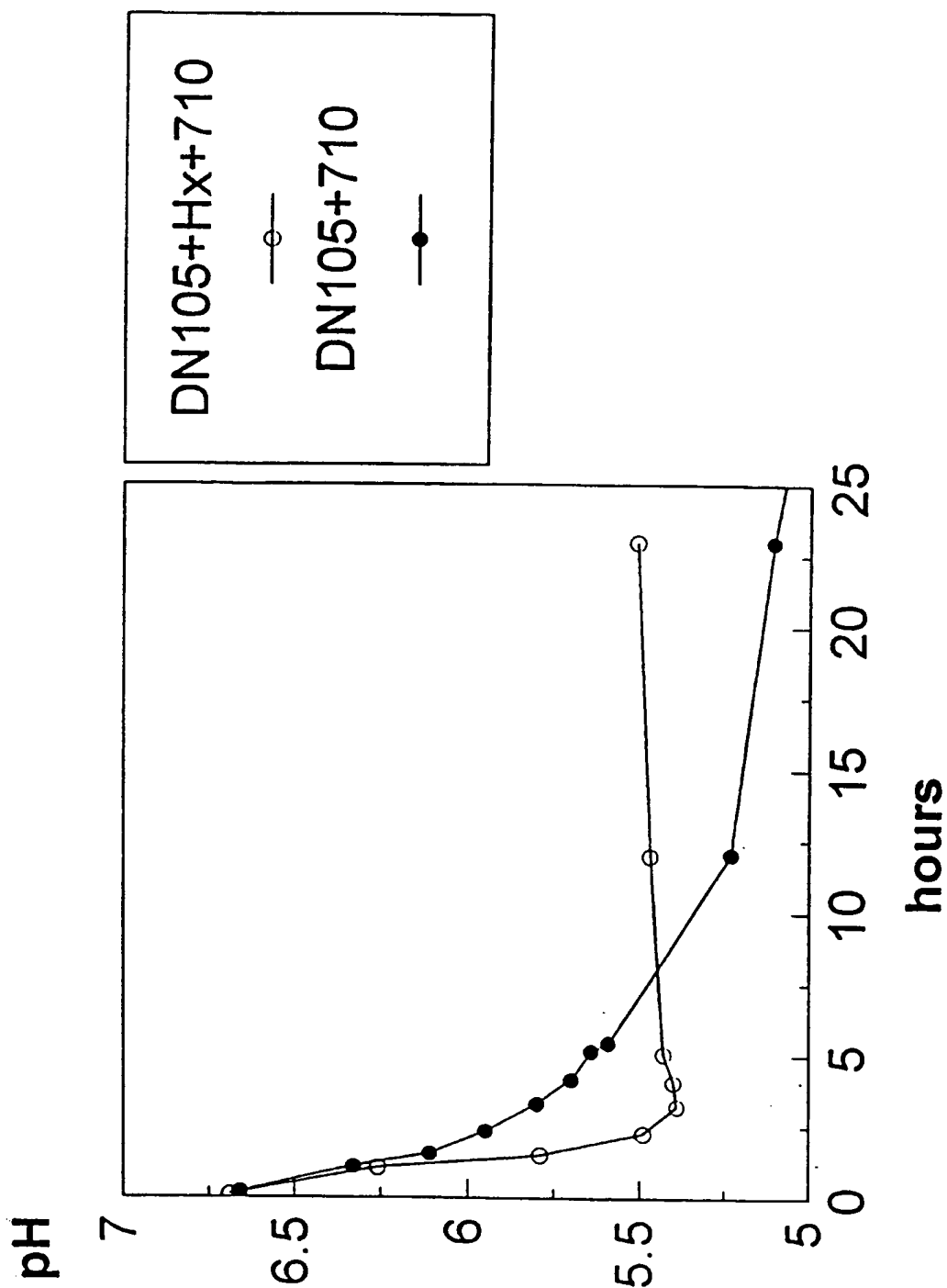
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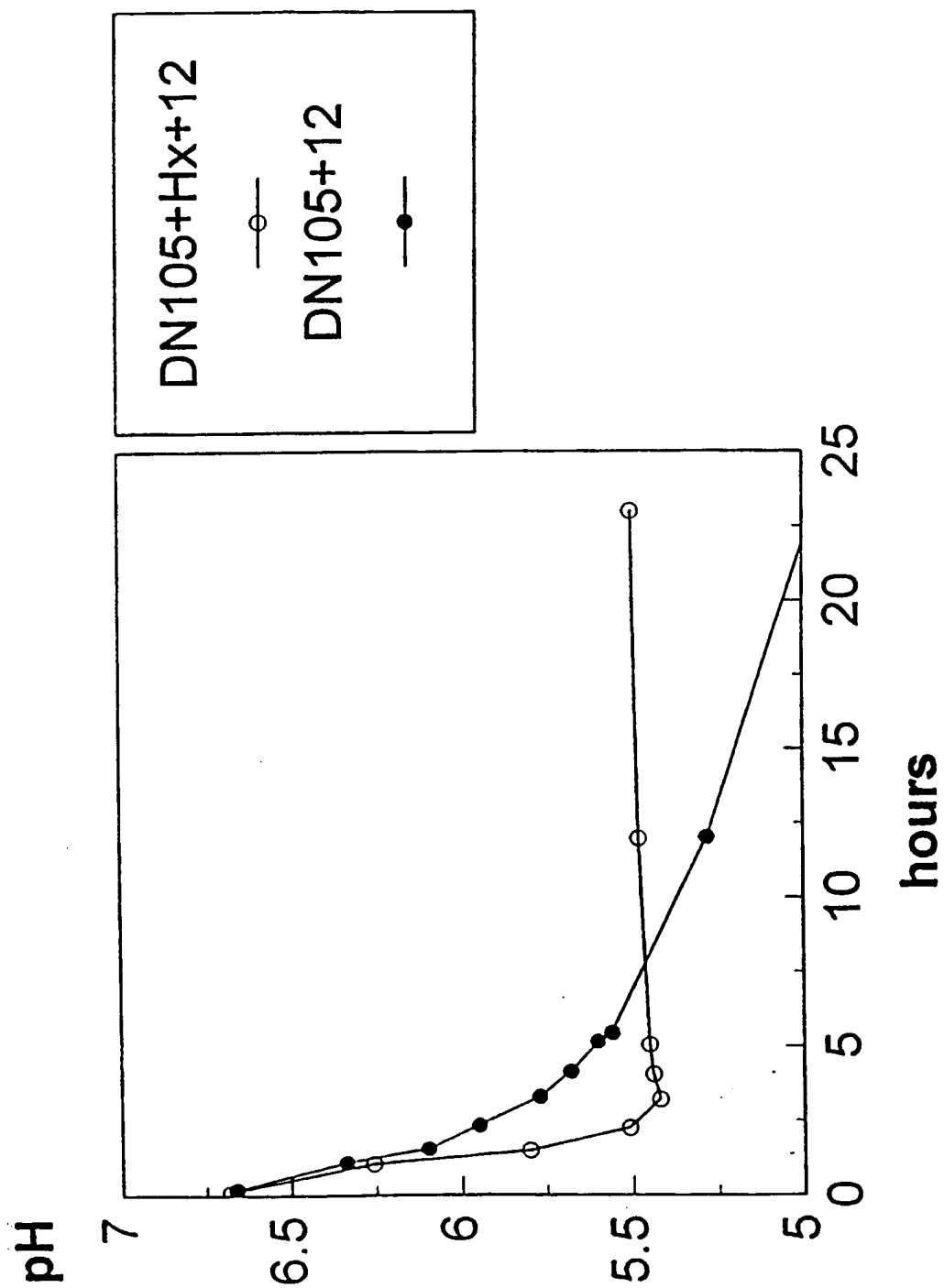
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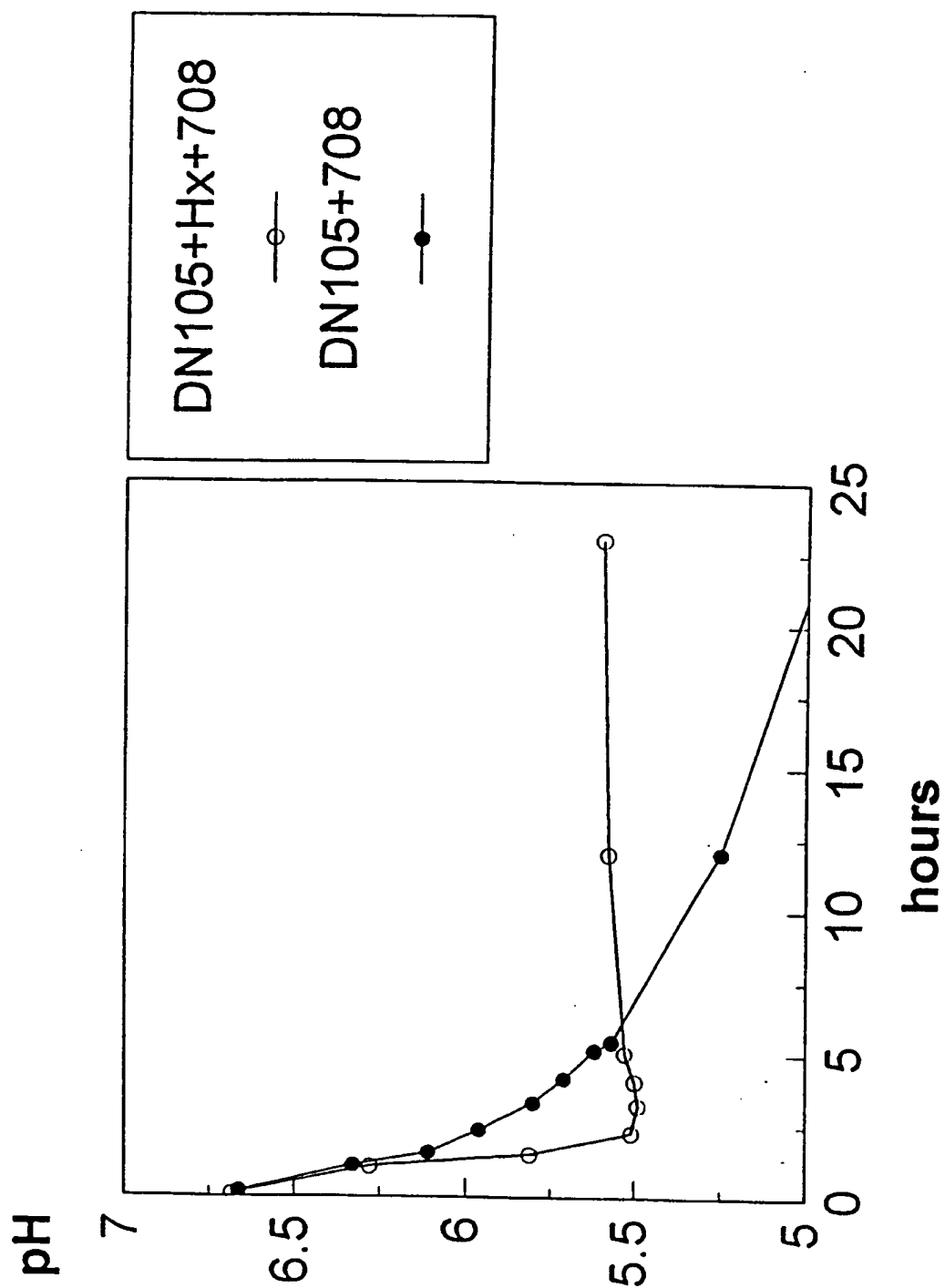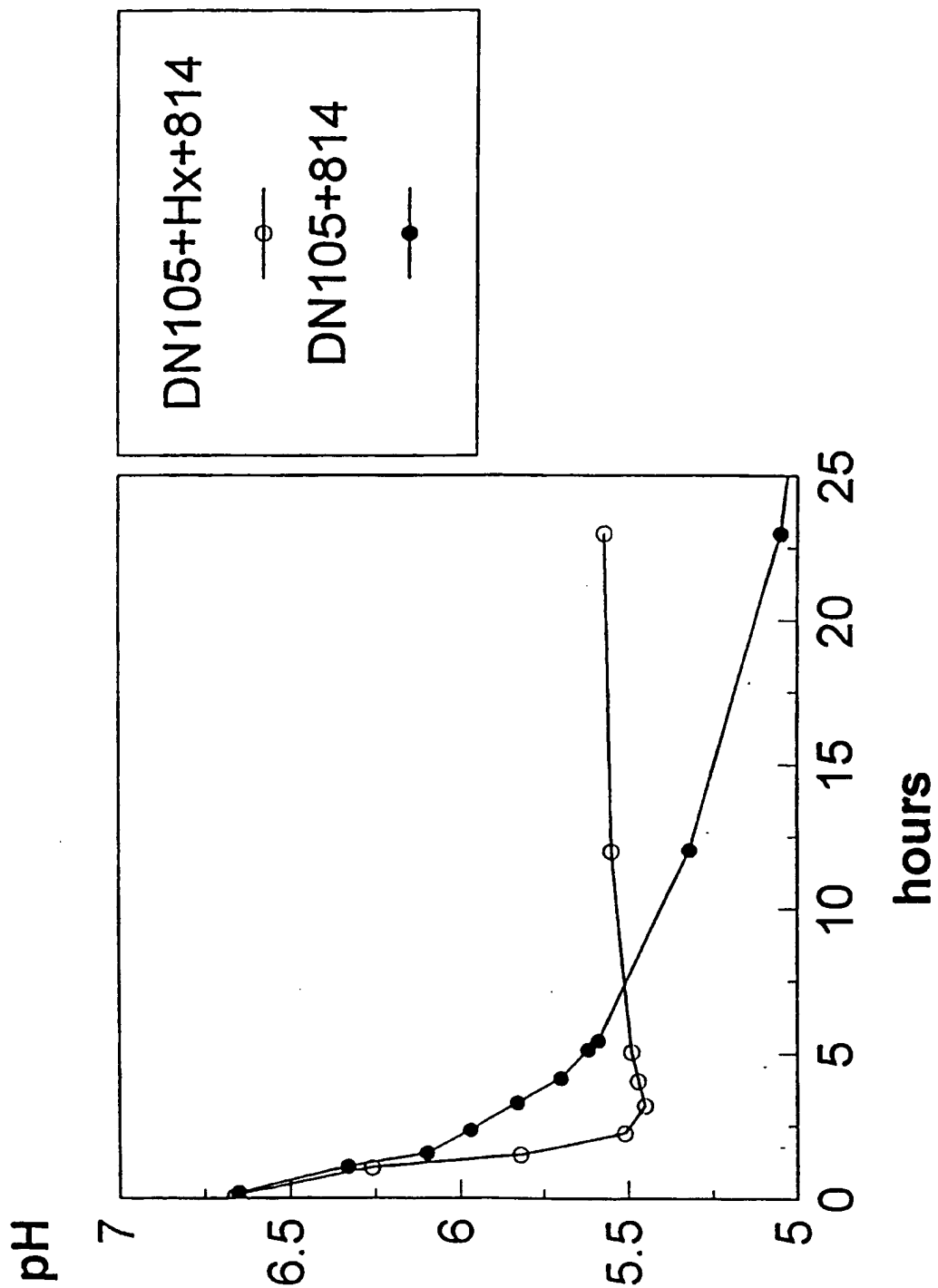
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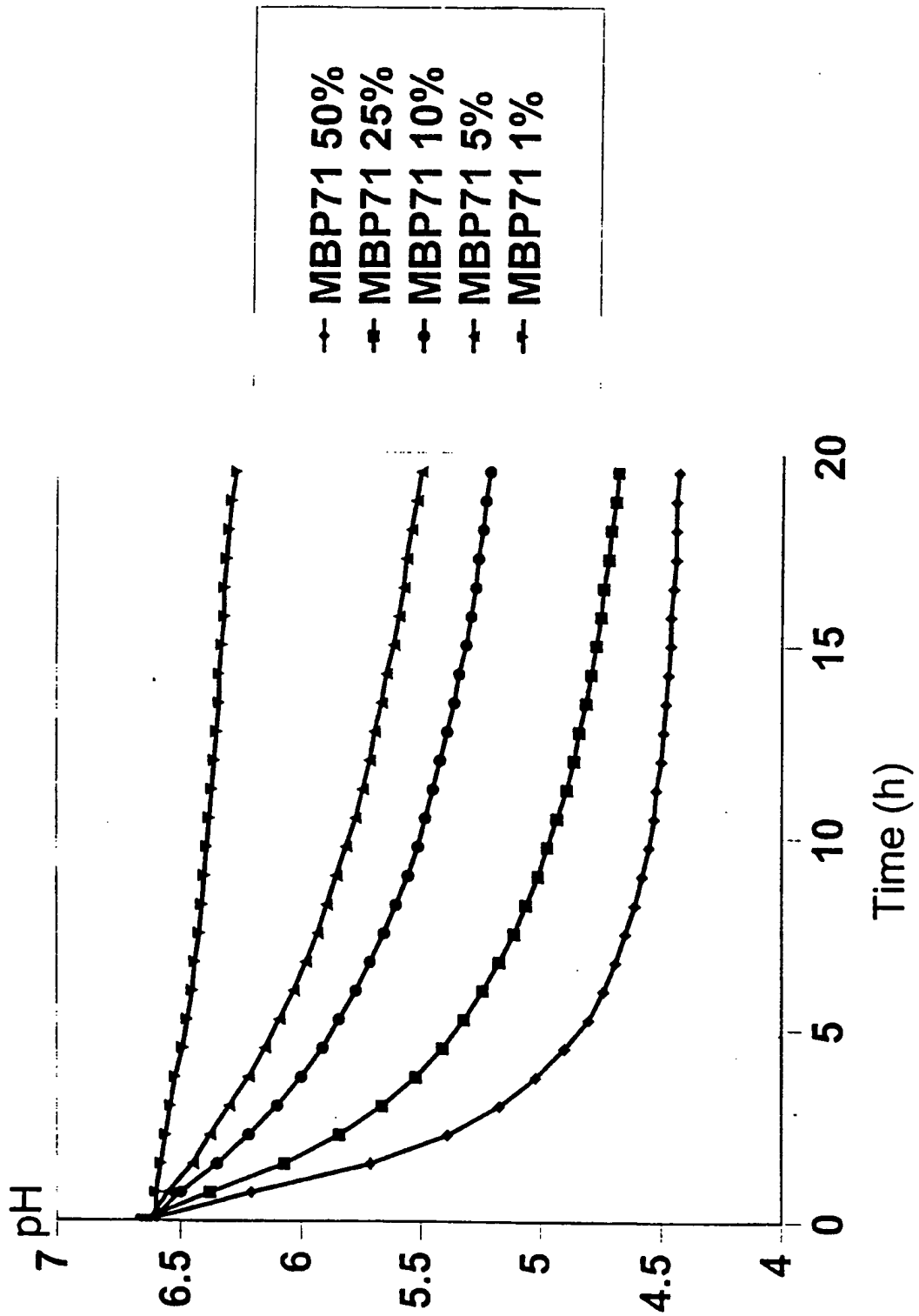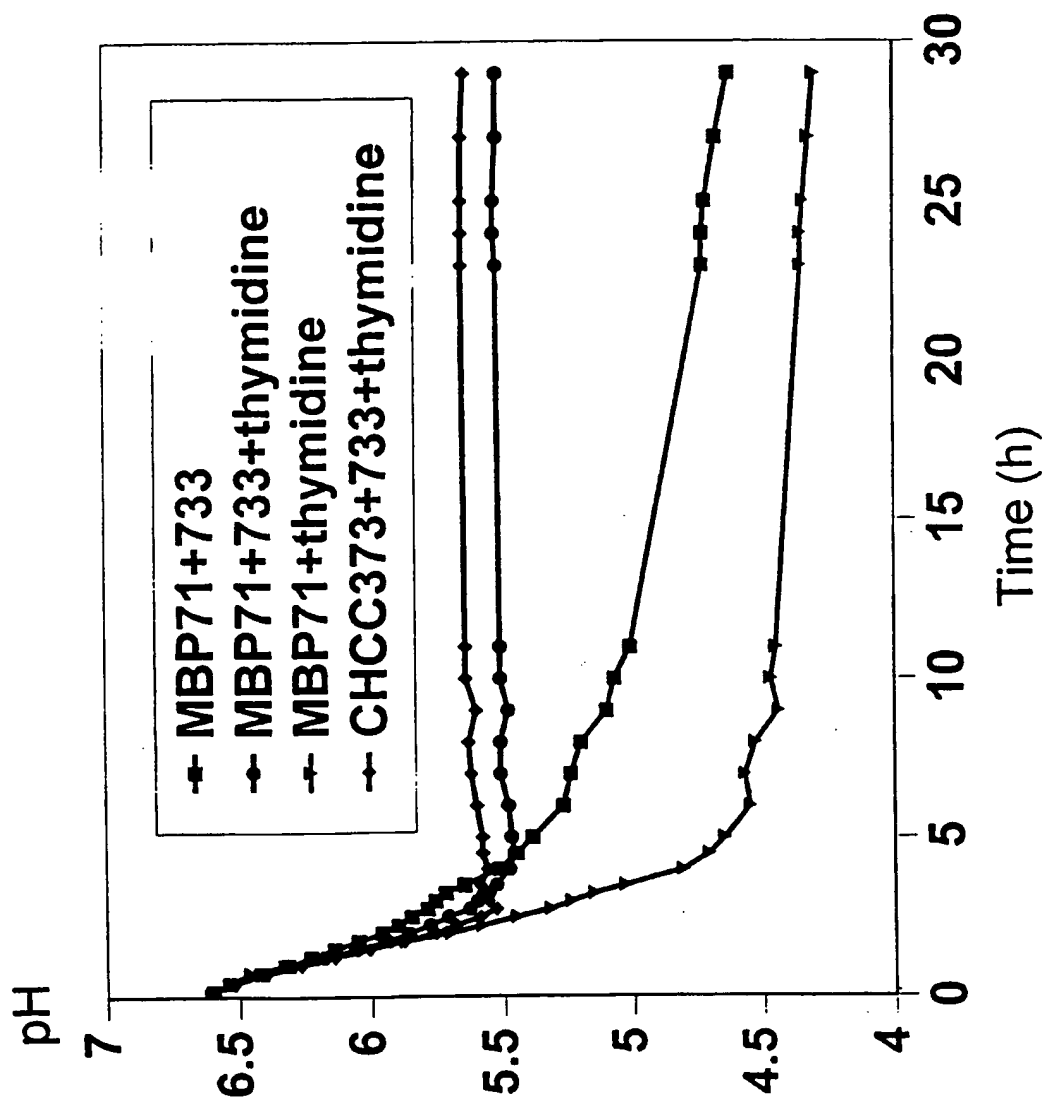
Fig. 4

Fig. 5

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